

# Neuronal Migration and Glial Remodeling in Degenerating Retinas of Aged Rats and in Nonneovascular AMD

Robert Sullivan,<sup>1</sup> Philip Penfold,<sup>2</sup> and David V. Pow<sup>1</sup>

**PURPOSE.** To demonstrate structural and immunocytochemical changes associated with light-induced degeneration in albino rat retinas and human AMD retinas.

**METHODS.** Retinas from Wistar rats aged 3, 6, or 10 months were examined by immunocytochemistry, with antibodies to neuronal and glial markers. Results were compared with human nonneovascular AMD retinas.

**RESULTS.** In aging rat retinas, many photoreceptors were lost in response to normal ambient light exposure. Photoreceptor loss was preceded by loss of RPE cells. Müller cells extended processes through gaps in Bruch's membrane, into the choroid. Immunolabeling for  $\gamma$ -aminobutyric acid (GABA), the glycine transporter Glyt-1, and the rod bipolar cell marker PKC revealed the presence of numerous neuronal somata and processes that appeared to have migrated into the choroidal region. Processes of presumptive ganglion cells remodeled and stratified in the choroid, where strong labeling for synaptic vesicle antigens was present. Myelination of retinal ganglion cell axons was also observed, especially in the peripheral retina. In AMD retinas, glial rearrangement and displacement of neurons suggestive of their migration were also observed.

**CONCLUSIONS.** In response to loss of RPE and photoreceptor cells, adult retinal neurons migrate out of the retina along remodeled processes of Müller cells. The presence of synaptic vesicle antigens suggests the formation of new synapses between migrating neurons. The myelination is probably due to the ingress of Schwann cells from the sclera. The presence of some similar changes in human AMD retinas suggests that these findings are of broad significance for determining the likely events in transplantation of neurons in the human retina and elsewhere. (*Invest Ophthalmol Vis Sci.* 2003;44:856–865) DOI:10.1167/iovs.02-0416

Anatomic observations on degenerative lesions of the retina frequently focus on the primary pathologic features such as loss of photoreceptors. All genetically based retinal degenerations lead to photoreceptor dysfunction and many lead to the death of both rod and cone photoreceptors.<sup>1</sup> However, loss of photoreceptors and the disruption of the RPE are clearly only parts of the cascade of events that ensue. Other down-

stream neuronal elements are also likely to be affected, either directly, because of factors such as loss of afferent synaptic inputs, or indirectly. Thus, for example, in the Royal College of Surgeons (RCS) rat model of retinal dystrophy, neuronal architectural changes may be evoked because of changes in the vasculature,<sup>2</sup> which may influence oxygen and metabolic substrate availability. An example of cascading changes in the organization of the neural retina is evident in studies of the cat retina, which have shown that extensive neural remodeling, especially in the inner nuclear layer, can occur in response to retinal detachment.<sup>3</sup> Such remodeling is presumably a downstream consequence of impaired photoreceptor survival, possibly because factors such as pigment epithelium-derived growth factor (PEDF) play a role in photoreceptor survival.<sup>4</sup> Similarly in human AMD the loss of photoreceptors within the lesion is thought to be associated with or possibly induced by changes in the retinal pigment epithelium or even in Müller cells.<sup>5,6</sup> Such changes may precede the reorganization of other retinal neurons and retinal glial cells.<sup>7</sup> A variety of models exist that cause photoreceptor degeneration, due to genetic lesions,<sup>8</sup> chemical lesions,<sup>9</sup> or photic insults.<sup>10</sup> Many lesions, such as the degeneration seen in the RCS rat,<sup>11</sup> are evoked during a relatively short period, typically a matter of a few weeks. Light has frequently been used to lesion the rat retina, especially in nonpigmented rodent species. Short periods (usually between 24 hours and 10–14 days) of bright illumination, high-energy blue-light illumination or sustained illumination can cause rapid degeneration of the rat retina.<sup>12–15</sup> Such exposure is also thought to cause rapid retinal degeneration, due to formation of very high levels of oxidative species, which quickly damage the retina.<sup>16,17</sup> Because of the rapid kinetics of these high-intensity- or sustained-light lesions, it is likely that little time may be available for secondary remodeling events to occur.

It has been proposed (albeit rather contentiously) for many years, that light is a causal agent in the degeneration of human retinas in AMD,<sup>18–20</sup> although this must be balanced against other possible epidemiologically defined risk factors, such as age, smoking, and genetic predisposition.<sup>21</sup>

Clearly, any human degeneration is slow when compared with the time frame for high-intensity light damage in rodent models. Accordingly, we have examined a well-known and consistently reproducible (but surprisingly little studied) model, which is the age-dependent, ambient light-induced degeneration of the albino rat retina.<sup>22,23</sup> In this model, degeneration occurs slowly, in response to exposure to normal ambient light levels (such as are typically found in any animal house). Such exposure, in our hands, produced a characteristic and progressive loss of photoreceptors. We compared the results obtained in this model with the results obtained in examining human retinas of deceased donors, in which there has been a clear diagnosis of AMD.

## MATERIALS AND METHODS

Male Wistar rats were maintained in our school animal house under normal animal house lighting conditions. Typical light intensity levels

---

From the <sup>1</sup>Department of Physiology and Pharmacology, School of Biomedical Sciences, University of Queensland, Brisbane, Australia; and the <sup>2</sup>Department of Clinical Ophthalmology, University of Sydney, Save Sight Institute, Sydney, Australia.

Supported by National Health and Medical Research Council of Australia Grants 210127 and 102448 (DVP).

Submitted for publication April 26, 2002; revised June 24, 2002; accepted July 9, 2002.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Robert Sullivan, School of Biomedical Sciences, Department of Physiology and Pharmacology, University of Queensland, Brisbane 4072, Australia; r.sullivan@mailbox.uq.edu.au.

in cages were measured with a standard handheld photography light meter. Light intensity within the cages was typically 750 lux. Animals were maintained on a 12-hour light–dark cycle and had access to food and water ad libitum. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with approval from the University of Queensland Group 1 Animal Ethics Committee.

Comparative observations were also made on human retinas ( $n = 6$ ) with demonstrable dry-form AMD lesions, some of which were prepared for a previous study.<sup>24</sup> The early stages of age-related macular degeneration are characterized by drusen and RPE pigmentary abnormalities that are designated by the term age-related maculopathy (ARM).<sup>25</sup> Diagnosis was made on this basis. Human tissues were obtained from donors with appropriate permission from the University of Queensland Human Ethics Committee and the Princess Alexandra Hospital Ethics Committee and in accordance with the Declaration of Helsinki.

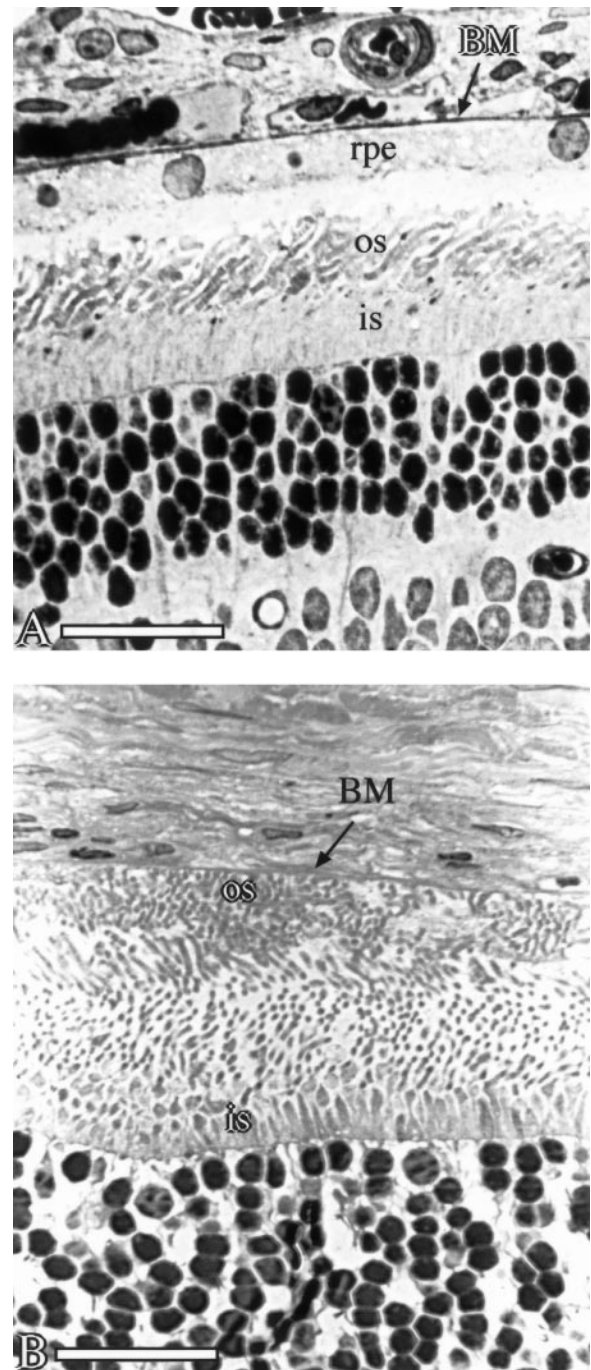
### Rat Tissues

Eyes were obtained from Wistar rats aged 3, 6, or 10 months. At each age, eyes of five animals were examined. A further two animals that were exposed to reduced light levels (250 lux) for 10 months were also examined.

Animals were killed by an overdose of pentobarbital sodium (100 mg/kg) administered by intraperitoneal injection. Eyes were enucleated, and the anterior portion of each eye, including the lens was removed. The resultant eyecups were fixed by immersion in 4% paraformaldehyde or 2.5% glutaraldehyde, both in 0.1 M phosphate buffer (pH 7.2). Glutaraldehyde-fixed eyes were embedded in epoxy resin according to standard protocols<sup>26</sup> and 0.5- $\mu$ m-thick semithin sections cut with an ultramicrotome. Semithin sections were either stained with toluidine blue, silver stained to demonstrate myelin,<sup>27</sup> or immunocytochemically labeled to detect  $\gamma$ -aminobutyric acid (GABA), which is a marker of GABA-containing neurons. The GABA antiserum has been extensively characterized.<sup>26</sup> The antiserum was used at a dilution of 1:300,000 in phosphate-buffered saline (pH 7.2) containing 0.5% bovine serum albumin (PBS-BSA; Sigma, Castle Hill, Australia), and immunolabeling of semithin sections was performed by standard methods, as described in our previous studies.<sup>26</sup> Primary antibody binding was detected with a biotinylated secondary antibody against rat immunoglobulins (Amersham Pharmacia Biotech, Little Chalfont, UK) at a dilution of 1:300 in PBS-BSA. Labeling was then performed with streptavidin-biotin horseradish peroxidase (HRP) complex (Amersham Pharmacia Biotech) at a dilution of 1:300 in PBS-BSA, with 3,3'-diaminobenzidine (DAB) used as the chromogen.

To demonstrate the location of multiple classes of neurons in microtome-cut sections (Vibratome; Ted Pella, Irvine, CA) a panel of antibodies directed against a variety of selective neuronal markers was used, including a rabbit polyclonal antibody against Glyt-1, which was raised in this laboratory and used at a dilution of 1:5000<sup>28</sup>; mouse monoclonal protein kinase C (PKC) purchased from Sigma and used at a dilution of 1:2000; rabbit polyclonal antibody against paraformaldehyde-conjugated glutamate, raised in this laboratory<sup>28</sup> and used at a dilution of 1:5000; and a monoclonal antibody to SV2 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), which was used at a dilution of 1:20. In the normal retina, the glycine transporter Glyt-1 is a distinctive marker of glycinergic amacrine cells,<sup>28,29</sup> and PKC is a marker of rod bipolar cells. Glutamate, is not specific for any single neuronal class, but in this study was used to illustrate cell populations such as retinal ganglion cells, whereas the specific synaptic vesicle marker SV2 was used to demonstrate the localization of putative synaptic structures.

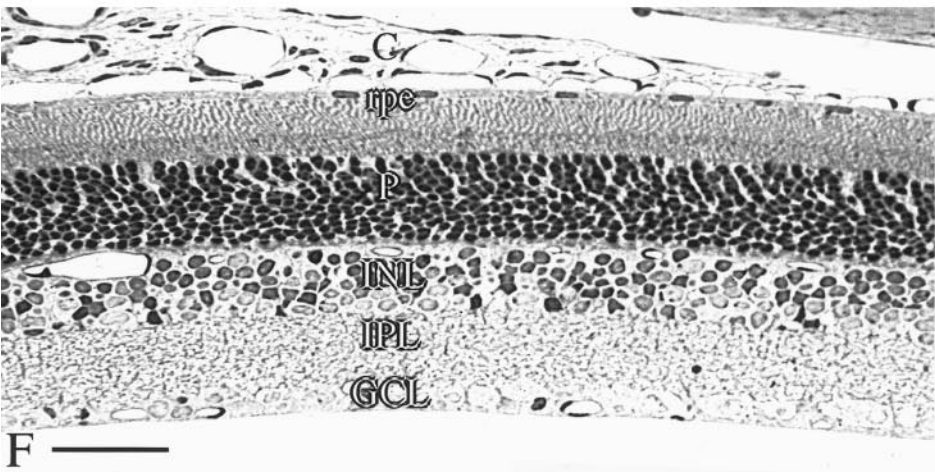
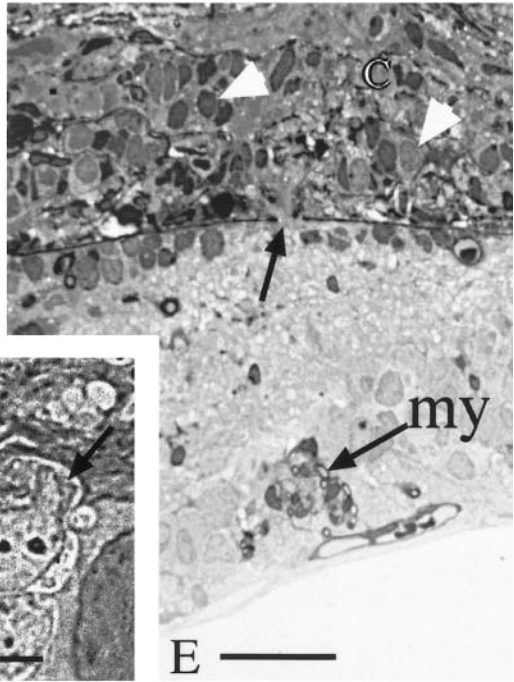
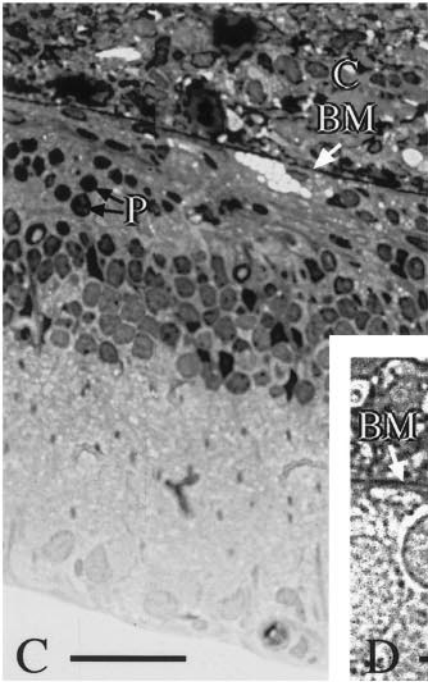
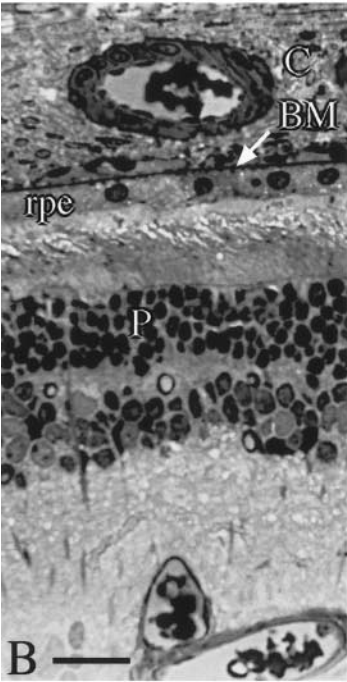
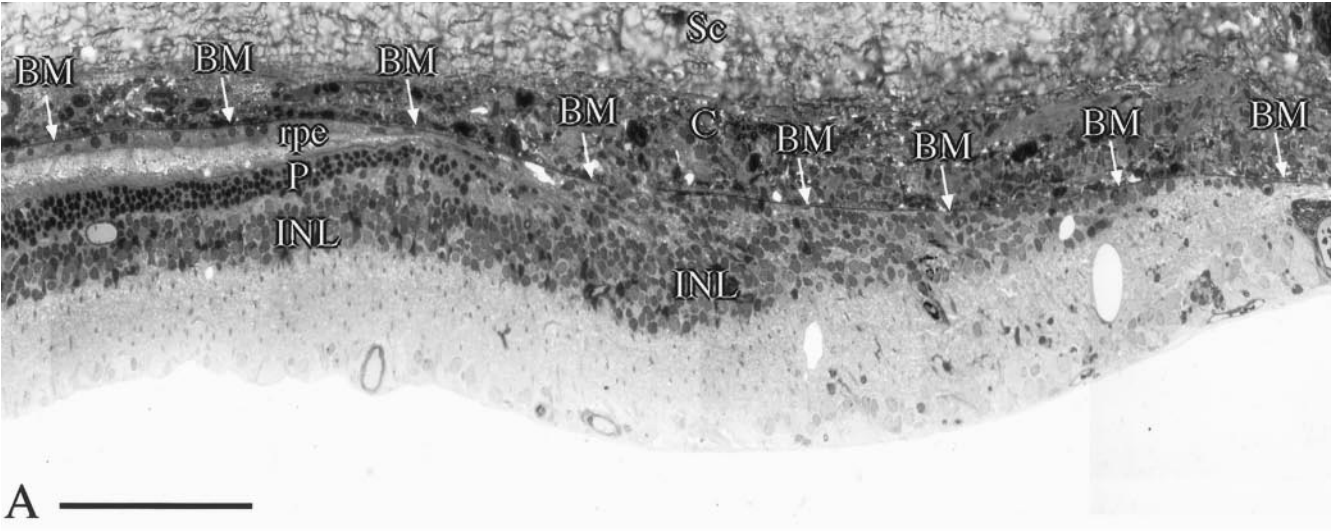
To immunolabel for Müller cells, two markers were used: the glutamate transporter GLAST which, in the retina, is a selective marker of Müller cells,<sup>30</sup> and glial fibrillary acidic protein (GFAP), which can label retinal astrocytes and Müller cells. Polyclonal GLAST antisera were raised in this laboratory<sup>30</sup> and used at a dilution of 1:40,000. The



**FIGURE 1.** Semithin transverse sections of 6-month-old rat eye 1 to 2 mm from the optic nerve head, stained with toluidine blue. (A) Region of retina where the photoreceptors, retinal pigment epithelium, and Bruch's membrane were intact. (B) Region where retinal pigment epithelium had been lost; photoreceptor outer segments directly appose Bruch's membrane (arrow). BM, Bruch's membrane; is, photoreceptor inner segments; os, photoreceptor outer segments; rpe, retinal pigment epithelium. Scale bars, 25  $\mu$ m.

monoclonal antibody to GFAP was obtained from Sigma and used at a dilution of 1:5000. Additional sections of optic nerve, sciatic nerve, brain, and retina were labeled with monoclonal Rip antibody (Developmental Studies Hybridoma Bank) used at a dilution of 1:20. Rip is a selective marker for oligodendrocytes.<sup>31</sup> Labeling was revealed either by immunoperoxidase techniques with a biotinylated secondary antibody (dilution 1:300; Amersham Pharmacia Biotech) followed by





streptavidin-HRP complex (dilution 1:300; Amersham Pharmacia Biotech), with DAB used as a chromogen, or with Texas red or FITC-conjugated secondary antibodies (Amersham Pharmacia Biotech at a dilution of 1:200).

Immunocytochemical control experiments were performed for all markers. For the polyclonal antisera, the control consisted of replacing the immune sera with preimmune sera from the same animals. For monoclonal antibodies the control consisted of substituting purified mouse IgG (Sigma) at a concentration of 10  $\mu$ g/mL in PBS-BSA, in place of the specific monoclonal antibody. Identical immunocytochemical control antibodies were also used for human tissues.

## Human Tissues

Human eyecups were fixed with 4% paraformaldehyde for 1 hour. Paraformaldehyde-fixed human eyecups were embedded in 4% agar and cross sections of the eyecups cut on a microtome (Vibratome; Ted Pella) at a thickness of 50  $\mu$ m. Sections were immunocytochemically labeled for either GFAP or the glycine transporter Glyt-1.

## Light Microscopy

Peroxidase-labeled sections were examined and photographed with a microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) equipped with Nomarski differential interference contrast optics, with images recorded on film (Ektachrome; Eastman Kodak, Rochester, NY). Slides were scanned into a computer (Slide Coolsan III Scanner, Nikon, Tokyo, Japan; imported into Photoshop 6, Adobe, Mountain View, CA). Fluorescently labeled sections were viewed with a confocal microscope (MRC 1040; Bio-Rad, Richmond, CA) and the digital files imported into a computer (Photoshop; Adobe). Digital files were minimally manipulated with changes made only in brightness and contrast. Composite image files were then created (Macromedia, Inc., Kew, Victoria, Australia) and printed (Stylus 1270; Epson Seiko, Nagano, Japan).

## RESULTS

### Analysis of Retinas

**Three-Month-Old Rats.** At 3 months the histology of the Wistar rat retinas appeared to be typical of this rat strain, with no evidence of any perturbation of structure. The RPE was intact, Müller cells were not swollen, and there was no histological evidence of the presence of any dying photoreceptors.

**Six-Month-Old Rats.** By 6 months of age, subtle changes were evident in the architecture of the Wistar rat retina. Although most of the retina appeared normal (Fig. 1A), some small patches of degeneration of the RPE were evident, scattered across the retina (Fig. 1B). Associated with such lesions was evidence of swelling of Müller cells in the outer nuclear layer (as evinced by areas that were unstained by toluidine blue in semithin sections) and possible loss of some photoreceptors in small patches.

**Ten-Month-Old Rats.** By 10 months of age gross lesions were demonstrable in the eyes of all rats that were exposed to normal ambient lighting conditions (Figs. 2A–E). Such lesions were continuous and occupied between half and two thirds of the area of the retina. The areas that were spared were im-

mediately surrounding the optic nerve head and the periphery of the retina. There was no evidence of any selectivity of the lesions for nasal or temporal sides of the retina, nor for any dorsal–ventral gradient.

### Histologic Analysis of the Lesions

Figure 2A illustrates a typical lesion. A consistent observation in this study was that distal to the edge of the lesioned areas, the retinas appeared to be generally intact, with normal apposition between the photoreceptors and the RPE (Fig. 2B). However, in close spatial register there was an abrupt loss of both RPE cells and the photoreceptors at the edge of the lesion (Fig. 2C). Bruch's membrane (a five-layered membrane that forms a boundary between the RPE and the choroid)<sup>32</sup> remained as a residual entity that was readily stained by toluidine blue. At the lesion's edge the inner nuclear layer was broadened (Fig. 2A), suggestive of the cells in this layer's having pulled away from their neighbors. Occasional holes were evident in Bruch's membrane in this region. A frequent observation was the presence of cell bodies apparently in the process of traversing Bruch's membrane, as evinced by the distorted dumbbell shape of the nucleus of such cells and the presence of cytoplasm on both sides of the membrane (Fig. 2D). Further into the lesion, there was a conspicuous loss of cells in the inner nuclear layer (Fig. 2E). Concomitant with this loss was the appearance of a similar number of cell bodies in the overlying choroidal region (Fig. 2E). The size and staining properties of these cells was suggestive of their being cells that had been lost from the inner nuclear layer. Careful examination of Bruch's membrane revealed many holes in this basement membrane. Contiguous streams of cellular material, including what appeared to be processes of Müller glial cells, appeared to link the retinal compartment below Bruch's membrane with the choroidal compartment above the membrane.

### Aged Rats Maintained under Lower Light Conditions

Although the primary purpose of this study was to illustrate the capacity for remodeling of the degenerating retina, rather than the mechanism of initial lesioning, per se, we confirmed as expected that light was the initial causal agent in this degenerative process. Examination of retinas from 10-month-old rats maintained under lower light conditions revealed no evidence of the presence of any degenerative lesions or reorganization (Fig. 2F).

### Immunocytochemical Control Experiments

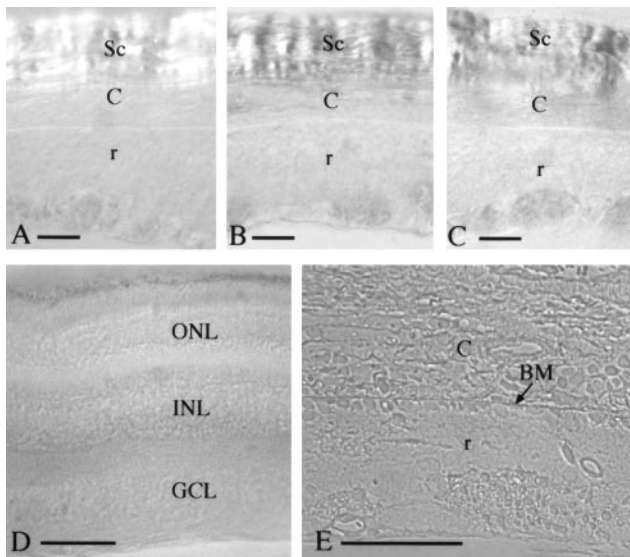
Immunocytochemical control experiments were performed for each of the antibodies used in this study (Fig. 3). In all cases no specific immunostaining was observed when the immune serum or monoclonal antibody was replaced by the preimmune serum or nonimmune mouse immunoglobulins.

### Immunocytochemistry for Astrocyte and Müller Cell Markers

Immunolabelling for GLAST (Figs. 4A, 4B) revealed an extraordinary and exuberant extension of Müller cell processes out of

**FIGURE 2.** Toluidine blue-stained section of an eyecup from a 10-month-old rat maintained under 750-lux illumination. Section was obtained 1 mm temporal to the optic nerve head and includes the sclera, choroid, and retina (A–E). (A) Low-magnification montage. At *left* (closest to the optic nerve head) is an intact region of retina; at *right* is a highly degenerated area. (B–E) High-magnification views of sections of (A) demonstrating relatively intact retina (B) and a region at the edge of the lesion where most photoreceptor somata had been lost (C). (D) A cell body is shown (*arrow*) that may have been in the process of translocating through Bruch's membrane. In regions with severe degeneration (E), holes were present in Bruch's membrane (*arrows*) and many neuronal somata (*arrowheads*) were present in the choroidal region. Darkly stained myelin was present. Conversely, in the retina of a 10-month-old rat maintained under low light conditions, there was no evidence of degeneration (F). BM, Bruch's membrane; C, choroid; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; my, myelin; P, photoreceptors; rpe, retinal pigment epithelium; Sc, sclera. Scale bars: (A, F) 100  $\mu$ m; (B, C, E) 20  $\mu$ m; (D) 5  $\mu$ m.





**FIGURE 3.** Immunocytochemical control sections. Microtome sections of 10-month-old degenerate rat retinas (A–C) or human retina (D) immunoperoxidase labeled with preimmune sera to GLAST (A), Glyt-1 (B), or purified nonimmune mouse immunoglobulin (C, D) in place of a specific mouse monoclonal antibody. Semithin, resin-embedded sections of 10-month-old degenerate rat retina were also labeled with preimmune sera for GABA (E). No specific labeling was present. *Arrow:* Bruch's membrane. C, choroid; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; r, retina; Sc, sclera. Scale bars, 50  $\mu$ m.

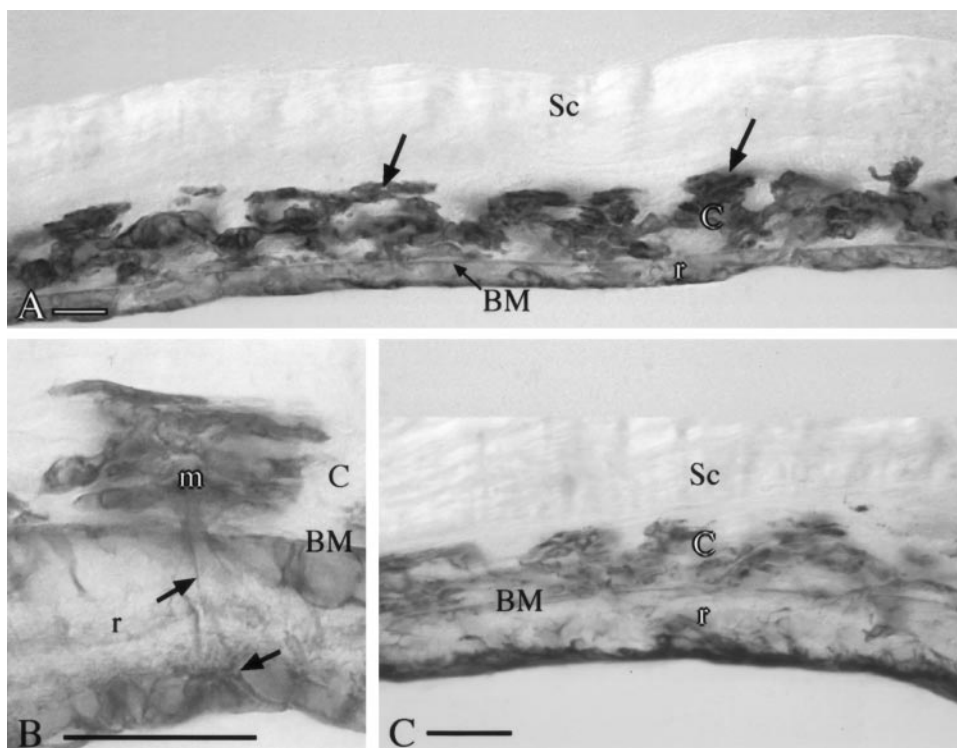
the retina and into the choroid. The Müller cell processes extended in a tree-trunk-like manner through holes in Bruch's membrane (which was readily identified with differential interference contrast optics) and then branched in the choroidal region to form a compact treelike structure with horizontally

stratified branches. Pale elliptical or pear-shaped gaps within the Müller cell branches appeared to represent crypts that contained unstained neuronal somata. GFAP staining (Fig. 4C) revealed a similar pattern of effusive outgrowth of the Müller cells. However, because GFAP is not expressed at the surface of the cell, it did not give as complete a picture of the Müller cell processes and their relationships to neurons as was evident with GLAST labeling.

### Neuronal Markers

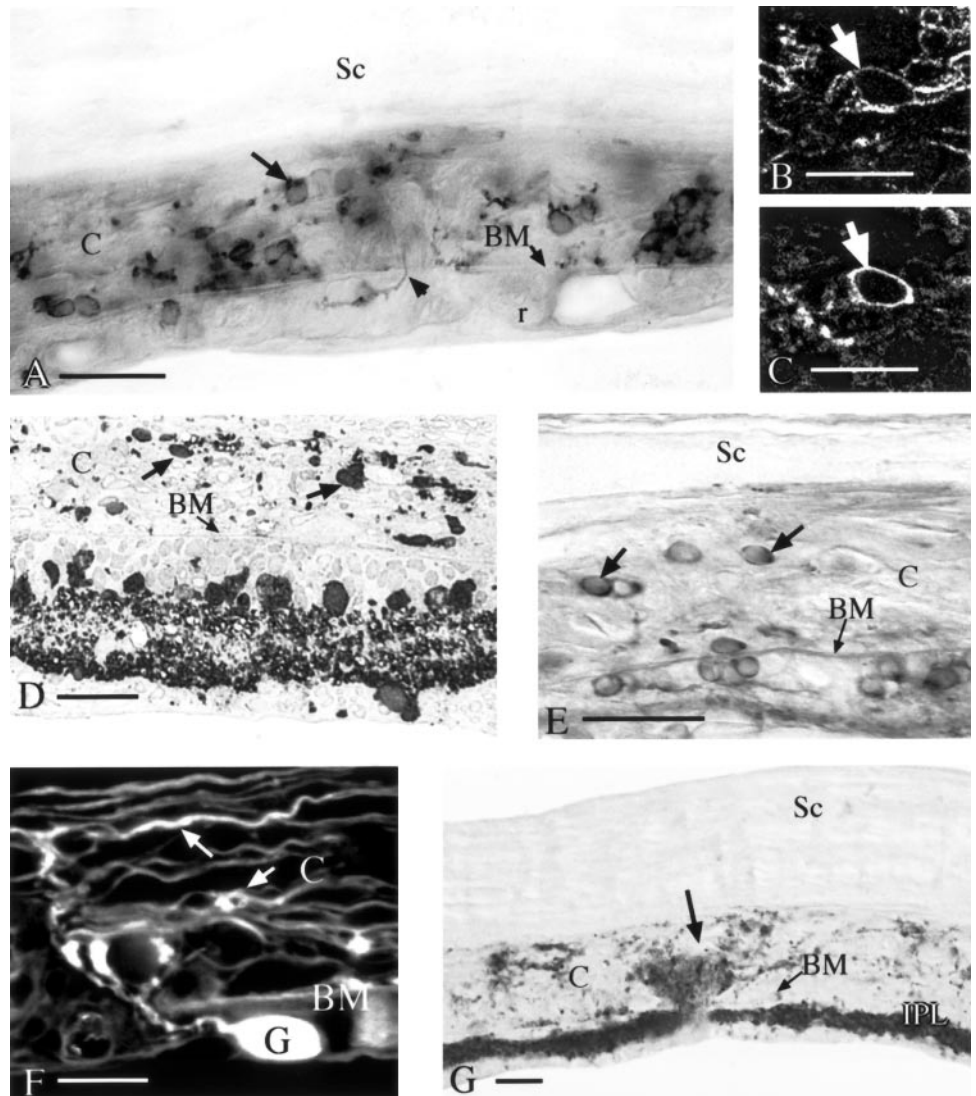
Immunolabelling for the glycine transporter Glyt-1, which is a robust marker for glycinergic amacrine cells in normal retinas of rats and humans,<sup>24,28,29</sup> revealed abundant labeling for this marker in neurons that were localized to the choroidal region (Fig. 5A) as well as those remaining within the retina. Double labeling for GLAST and Glyt-1 revealed (Figs. 5B, 5C) that within the choroidal region the Glyt-1-immunoreactive cell bodies of the ectopically positioned glycinergic amacrine cells were located within GLAST-immunoreactive Müller cell crypts.

Immunolabelling of semithin, resin-embedded sections for GABA, which is a reliable marker for GABAergic neurons revealed consistently strong labeling for this neurotransmitter, both within the retina and in cells located within the choroid (Fig. 5D). Labeling was identified, not only in presumptive neuronal somata but also in many fine punctate structures, which we interpreted as neuronal processes or terminals. Similarly, immunoreactivity for PKC (Fig. 5E) was evident in many cells in the choroidal region, suggestive of the migration of rod bipolar cells out of the retina and into the choroidal region. In all cases, the ectopically located neurons appeared to maintain a close association with the Müller cells, as revealed by dual immunofluorescence labeling. Ectopically located neurons were localized to the elliptical or pear-shaped crypts formed by the Müller cell processes. Not all neuronal types appeared able to migrate. Retinal ganglion cells appeared to remain in the residual ganglion cell layer. However, the inner plexiform layer



**FIGURE 4.** Microtome section through a degenerate region of 10-month-old rat retina immunoperoxidase labeled for GLAST (A, B) or GFAP (C). All sections were approximately 2 to 3 mm temporal to the optic nerve head. GLAST-immunoreactive processes of Müller cells (A, B) extended out of the retina into the overlying choroid. *Arrows:* Müller cells exhibiting a treelike morphology in the choroidal region. The trunk process of a Müller cell (B) extended through the retina (*arrows*), but lateral processes were not evident within the retina. GFAP labeling (C) were similar to the labeling observed with GLAST. BM, Bruch's membrane; C, choroid; m, Müller cell; r, retina; Sc, Sclera. Scale bars, 50  $\mu$ m.

**FIGURE 5.** A 10-month-old degenerate rat retina. (A–C, E–G) Microtome sections; (D) a semithin, resin-embedded section. (A, D, E, G) Immunoperoxidase-labeled sections; (B, C, F) immunofluorescence-labeled sections. All sections were approximately 2 mm temporal to the optic nerve head. (A) Glyt-1-immunolabeled glycinergic amacrine cells (*arrow*) were located in the choroid and immunoreactive cell processes (*arrowhead*) traveling between the retina and choroid, through gaps in Bruch's membrane (*arrowhead*). Double-labeled immunofluorescence confocal images of GLAST labeling (B) and Glyt-1 labeling (C) revealed that a Müller cell process surrounded a Glyt-1-immunoreactive neuron (*arrows*) located in the choroid. Immunolabeling for GABA (D) or protein kinase C (E) revealed that GABAergic and glutamatergic neurons may be ectopically located in the choroidal region. By contrast, the soma of a large glutamate-immunoreactive ganglion cell (F) did not migrate, but its dendritic processes were stratified (*arrows*) in the choroidal region. SV2-immunoreactive elements (G) streamed through a gap in Bruch's membrane and into the choroid (*arrow*). BM, Bruch's membrane; C, choroid; G, ganglion cell; IPL, inner plexiform layer; r, retina; Sc, sclera. Scale bars: (A, D, E, G) 25  $\mu$ m; (B, C, F) 10  $\mu$ m.



thinned out (due to the exodus of interneurons into the overlying choroidal region) so that the ganglion cells, which were revealed (albeit nonselectively) by intense immunolabeling for the retinal ganglion cell neurotransmitter glutamate, appeared to extend processes into the choroidal region (Fig. 5F). Such processes appeared to stratify in several horizontal strata similar to the strata formed by the branches of the Müller cells.

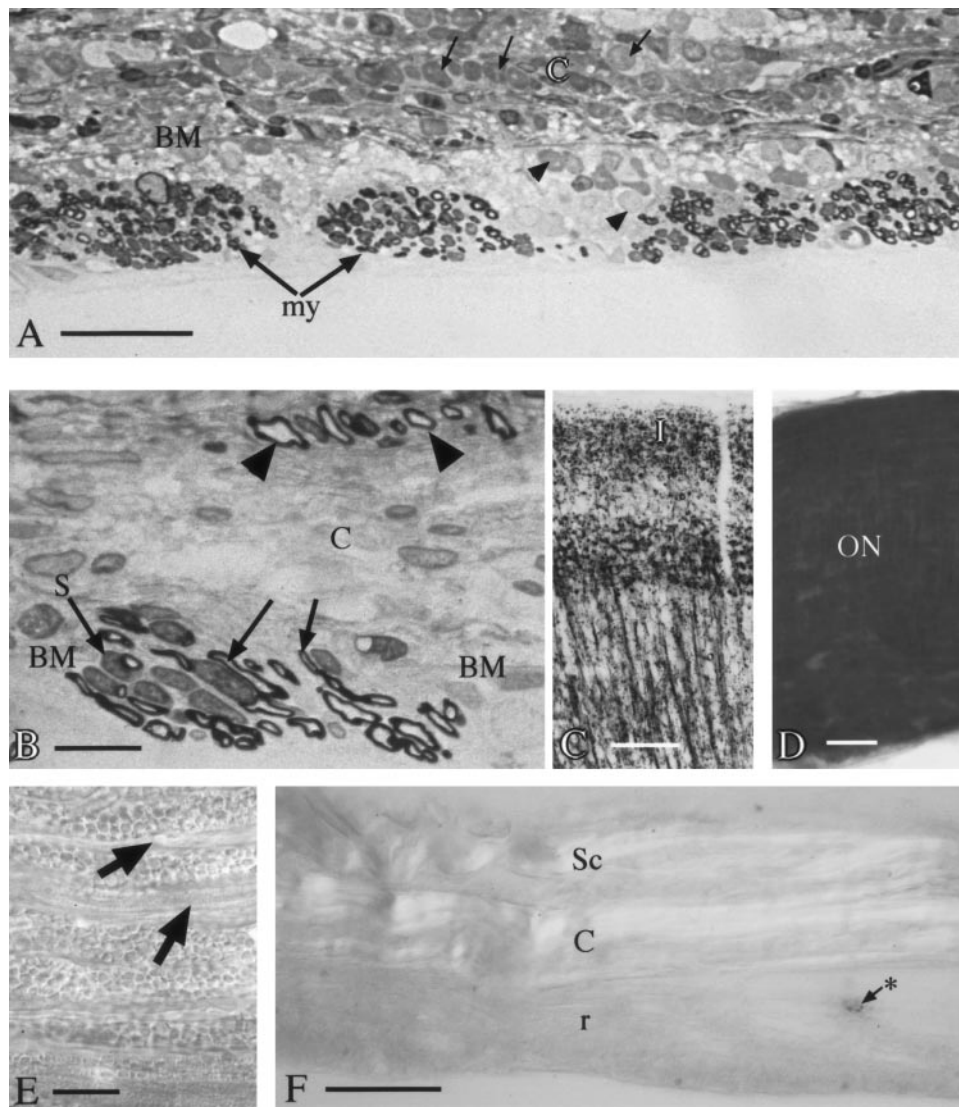
To investigate whether it was possible that the GABAergic, glycinergic, and glutamatergic neuronal types that were present in the choroidal region might be capable of forming synaptic connections, sections were immunolabeled for the synaptic vesicle marker SV2. Strong and consistent SV2 staining was evident in those areas of the choroid occupied by migrated neurons (Fig. 5G). In many cases streams of SV2-labeled elements were observed that extended along those Müller cell processes that protruded through holes in Bruch's membrane, between the retina with the choroidal region. By contrast, and in accord with innumerable previous studies, none of these neuronal markers was observed in the choroidal region of normal nondegenerate areas of the retina.

### Myelination

It was noted unexpectedly, initially in toluidine blue-stained sections and later in sections selectively stained with silver

to reveal myelin, that many axons in the nerve fiber layer of the retina were myelinated (Fig. 6A). Such myelination was not evident around the optic nerve head but was instead most evident in areas in the center of the lesions. Because retinal interneuron processes were not evident in such areas, it was assumed that the myelinated processes were those of retinal ganglion cells. The silver staining characteristics of the retinal myelin were identical with those observed around nearby myelinated axons traveling at the sclera-choroid interface (Fig. 6B). Cells that we interpreted as being Schwann cells each appeared to myelinate a single axon (a feature that distinguishes them from oligodendrocytes, which typically myelinate multiple axons; Fig. 6B). Immunolabeling with the Rip antibody, which is reported to be selective for oligodendrocytes, revealed strong labeling in oligodendrocyte-rich tissues, such as cerebral cortex (Fig. 6C) and optic nerve (Fig. 6D), whereas no demonstrable labeling was evident in sciatic nerve where the myelinating cells were Schwann cells (Fig. 6E). Similarly, no labeling was evident in those retinas that contained abundant myelin (Fig. 6F), suggesting that the myelinating cells in these retinas were Schwann cells, similar to those that myelinate the axons running at the boundary of the sclera and choroid.





**FIGURE 6.** Resin-embedded sections of 10-month-old rat retina 2 mm temporal to the optic nerve head were silver stained for myelin (A, B). Myelinated ganglion cell processes were darkly stained. In the choroid, numerous large somata (arrows) were present. At high magnification (B), somata of presumptive Schwann cells could be identified in association with myelinated axons. Additional myelinated axons were present at the boundary of the sclera and the choroid (arrowheads). (C–F) Microtome sections immunoperoxidase-labeled for Rip. Strong labeling was present in the cortex of 10-month-old rat (C). Layer 1 (I) is identified at the top of the image. In rat optic nerve (D), Rip staining was so intense that individual axons and oligodendrocytes could not be discriminated. Conversely, in sciatic nerve (E) no specific staining was evident. Arrows: longitudinally sectioned axon bundles. In a 10-month-old rat retina (F), no specific labeling was evident. Mast cells (arrow, \*) contained only endogenous peroxidases. BM, Bruch's membrane; C, choroid; my, myelin sheaths; ON, optic nerve; r, retina; S, Schwann cell. Scale bars: (A, C, F) 50  $\mu$ m; (B) 10  $\mu$ m; (D) 100  $\mu$ m; (E) 20  $\mu$ m.

### Human Nonneovascular AMD Retinas

We reexamined Glyt-1-labeled retinas from a previous study and additional GFAP-labeled sections, to determine whether any of the changes in retinal structure observed in the present study are also evident in human tissues.

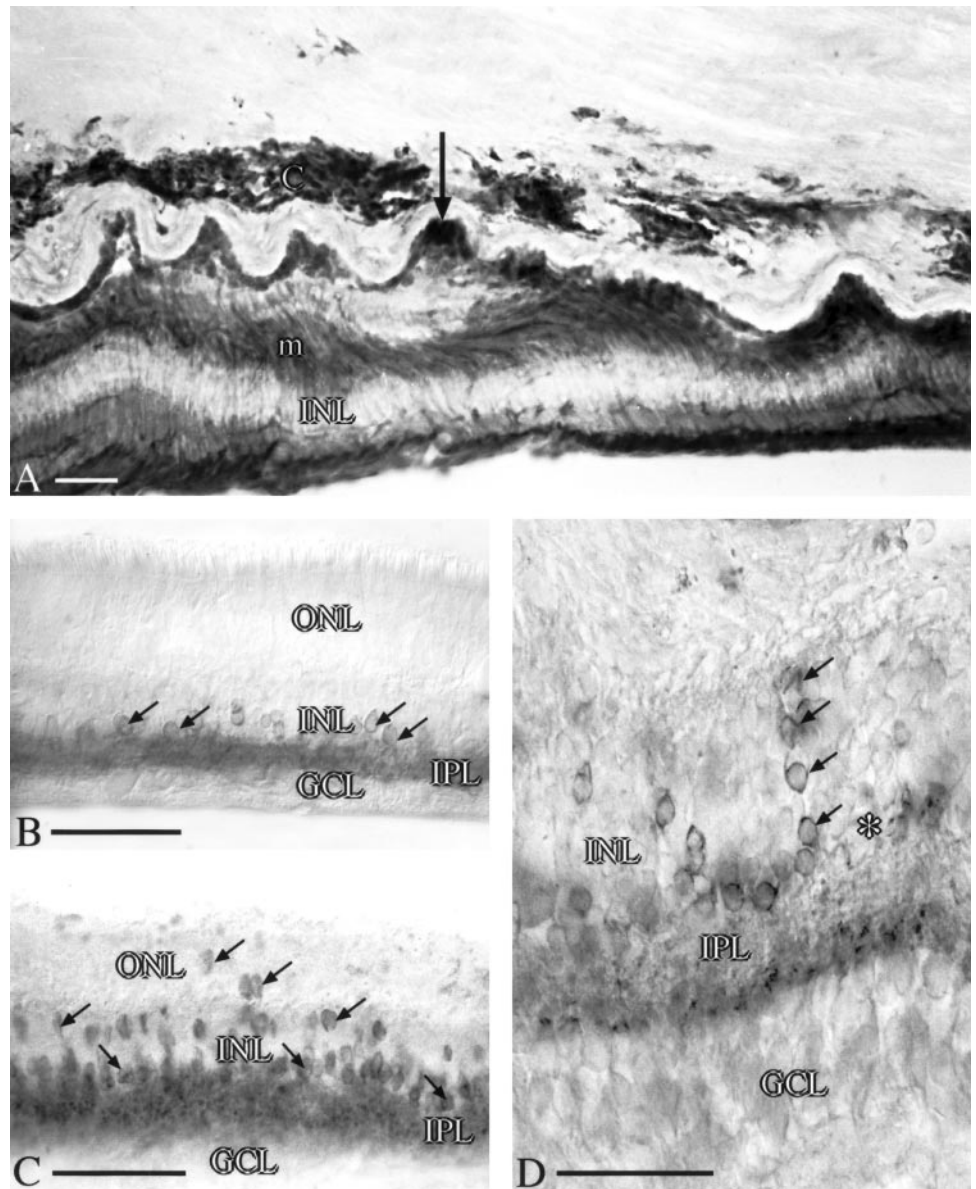
Within the area of degeneration, GFAP staining was intense, especially in the regions of the inner and outer end feet of the Müller cells. Numerous volcano-like eruptions of the Müller cells were observed (Fig. 7A). These protrusions of Müller cells were especially evident in regions where the pigment epithelium had been lost.

Glyt-1 is a marker for glycinergic amacrine cells. In nondegenerate areas of the AMD retinas outside the macular region, labeling was present in the plasma membranes of amacrine cells, the somata of which were normally located in the inner third of the inner nuclear layer (Fig. 7B), in accord with our previous studies of normal retinas.<sup>28</sup> However, within the lesions, especially where the volcano-like protrusions of Müller cells were present, we also observed Glyt-1-immunoreactive cells that were displaced into the outer part of the retina (Fig. 7C). Such displaced cells were observed in all the retinas studied, but the incidence was variable, suggesting it was dependent on the extent of the disease at the time of death of the eye donor. However, in all eyes studied, it was observed

that Glyt-1-immunoreactive amacrine cells were often clustered as columns of three to six cells (Fig. 7D), suggestive of their migration along a common conduit, possibly a radial glial cell process.

### DISCUSSION

In this study we made four distinct findings: (1) Light damage in the albino Wistar rat retina caused the slow and progressive formation of a typical lesion, with loss first of the RPE and then of the photoreceptors. (2) In the rat retina, Müller cells extended processes out of the retina through the subretinal space, and, through holes in Bruch's membrane, into the overlying choroid, where they formed multiple horizontally stratified branches. The Müller cell processes apparently serve as conduits along which retinal neurons then migrate out of the retina, into the choroid, where they may form synaptic connections. (3) In the rat retina, myelination of putative retinal ganglion cell axons was evident at sites distant from the optic nerve head, especially in areas that exhibited extensive fragmentation of Bruch's membrane. (4) Human nonneovascular AMD retinas showed evidence of anatomic remodeling, including migration of neurons and reorganization of Müller cell processes—the remodeling in the AMD retinas being similar in



**FIGURE 7.** Microtome sections of retina from a patient with a clinical diagnosis of dry AMD immunoperoxidase labeled for GFAP (A) or Glyt-1 (B–D). Numerous eruptions (arrow) of GFAP-immunoreactive Müller cells were evident (A). In a section 2 to 3 mm lateral to the lesion (B) immunoreactive amacrine cell somata (arrows) were located in a narrow band close to the inner plexiform layer. Within the lesion (C) amacrine cell somata (arrows) were located in a broad band that extended up into the residual outer nuclear layer. At high magnification (D) immunoreactive amacrine cell bodies (arrows) appeared in a columnar arrangement suggestive of their radial dispersion. Conversely, an adjacent region (\*) was depleted of any immunoreactive amacrine cells. C, choroids; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; m, Müller cell processes; OPL, outer plexiform layer. Scale bars, 50  $\mu$ m.

character to that observed in the rat, but of a more modest nature.

### Fragmentation of Bruch's Membrane in Rat Retina

In this study we demonstrate the fragmentation of Bruch's membrane, yielding many holes that serve as gateways for subsequent glial outgrowth and thence neuronal migration. Fragmentation of Bruch's membrane is a typical feature of AMD, with the severity of the AMD generally related to the extent of fragmentation.<sup>33</sup>

We did not find evidence in this model of any neovascularization. Blood vessels were never observed to extend between the choroid and the retina, per se, suggesting that processes such as neovascularization were not causal in the breakdown of Bruch's membrane in this instance.<sup>34</sup>

### Migration of Retinal Neurons

In the present study, we concluded that in rats there is a spatially defined zone at the edge of the lesions we describe where neurons are lost from the inner nuclear layer of the

retina and that there is a concomitant appearance of neurons in the choroidal region. We formed this conclusion, based both on our conventional histologic data and on the basis of the distribution of cellular elements that are immunolabeled with our panel of markers for neurons. Although no single marker can be assumed to confirm unequivocally the neuronal status of the cells that we observed in this study, the presence of multiple markers such as GABA and SV2, which are generally regarded as selectively identifying neurons, along with Glyt-1 and PKC, makes a strong case that we are indeed observing neurons that have migrated out of the retina into the overlying choroid.

Our finding of abundant neuronal migration was initially surprising, because a similar phenomenon has not been reported in rats exposed to short- or long-term light damage.<sup>10,13–16</sup> We suggest that this is fortuitous at two levels. First, we have studied a model that has an extended period of degeneration and therefore permits the processes of cellular reorganization and migration to occur. Second, it was fortunate that we took the unusual step of looking for neural markers in the choroidal region of the eye, because this would not be



undertaken in most studies. In view of the clear evidence of cellular elements traversing between the retina and the choroid through holes in Bruch's membrane, we conclude that the neurons that we observed in the choroidal region had migrated between these two anatomic compartments. We do not believe that the neurons appear in the choroidal region due to a process of *de novo* neurogenesis, because the areas of neuronal loss in the retina and neuronal gain in the choroid are in complete spatial register. Clearly, the concept that GABAergic, glycinergic, and glutamatergic neurons might be able to migrate *in toto* greatly extends the current ideas of nervous system plasticity. Classic views of plasticity emphasize changes in features such as dendritic arbors of neurons, but do not normally countenance the complete relocation of neurons. This view is surprising, given our current understanding of the way in which cells such as neuronal stem cells are able to migrate through the matrix of the nervous system. We propose that migration of neurons in response to changes in synaptic input may well be commonplace, but that without reference to precise borders such as the remnants of Bruch's membrane, such movements may be undetectable by conventional histologic means. In support of this view, we note that in unpublished studies we have observed similarly displaced cells at the edge of lesions in retinas of taurine-depleted cats which also exhibit an AMD-like lesion in the area centralis.<sup>35</sup> Accordingly, we suggest that the migration of retinal neurons in response to loss of functional synaptic inputs, regardless of the causal nature of the lesions, may be a common phenomenon among many species. Does neuronal migration occur in humans? Our data suggest that in the degenerating human retina neuronal migration does indeed occur, as evinced by the presence of glycinergic amacrine cells that are ectopically located in the outer nuclear layer at the margins of lesions. We have not, however, demonstrated a generalized further migration into the choroidal region, although some isolated instances of glial processes extending through the RPE were observed.

Do neurons that have migrated form demonstrable synapse-like structures? The current studies suggest that in the rat, SV2-immunoreactive synaptic vesicle clusters are present in the neuronal elements that had migrated into the choroid. Extensive punctate labeling was also observed along the course of Müller cell processes, as they traveled between the retina and the choroid. Such labeling may indicate either the presence of en passant-type synaptic varicosities or the formation of connections with other processes such as ganglion cell processes, which we have observed traversing this region. Although this is undoubtedly a prerequisite for synaptic interactions, more data are needed to confirm that connections are formed. Future studies may include an analysis of markers such as the postsynaptic density marker PSD 95, to verify whether demonstrable postsynaptic specialization is present, and electron microscopy. However, such studies were outside the scope of the present investigation.

### Müller Cell Processes

The finding, in this study of excrescent outgrowth of Müller cell processes in our rat model and more modest remodeling in the human AMD retinas has parallels several other studies. Retinal detachment can result in outgrowth of Müller cell processes into the subretinal space.<sup>3</sup> Similarly, other studies of AMD retinas have demonstrated that Müller cell processes may extend and adhere to the denuded Bruch's membrane in an attempt to maintain the blood-retina barrier.<sup>36-39</sup> We propose that neurons that migrate into the choroidal region of the rat eye may survive because of the unusual morphology of the Müller cells, which, by extensively wrapping the neurons, may provide a regulated environment that functions as a quasi blood-retina barrier.

### Myelination of the Retinal Ganglion Cell Axons within the Rat Retina

In this study, concomitant with the degeneration of the retina, abundant myelination of what we presume to be ganglion cell axons was evident. In the normal rat retina, myelination is not normally observed, presumably because the myelin would cause degradation of images, due to the high refractive index of the myelin, which causes light scattering. Myelination has previously been described in response to puncturing lesions from the sclera into the retina, which penetrate Bruch's membrane.<sup>40</sup> Perry and Hayes<sup>40</sup> suggested that because the lesion had to penetrate the sclera and the retina, the Schwann cells responsible for this myelination arise in the sclera and migrate along the lesion tracts into the retina. We suggest that Schwann cells were responsible for the observed myelination in this study. Schwann cells can generally be distinguished from oligodendrocytes because Schwann cells usually associate with only a single myelinated axon, whereas oligodendrocytes tend to wrap multiple axons. Our additional observations with the use of Rip antibody, which is reported to be a specific marker for oligodendrocytes rather than Schwann cells,<sup>31</sup> suggest that the myelinating cells are Schwann cells rather than oligodendrocytes. We base this conclusion on our observation that although oligodendrocytes are strongly labeled in brains and optic nerves of these animals, little if any labeling is observed in their sciatic nerves (which are myelinated by Schwann cells), and similarly there was no observable labeling with the Rip antibody in the retinas of these animals. What might the functional role be of this myelination? We know from earlier studies by Lawrence et al.<sup>41</sup> that Schwann cells, when introduced into the subretinal space, retard degeneration of the RCS rat retina, possibly because of secretion of trophic factors. We suggest that the myelination observed in this study may well be a secondary consequence of spontaneous recruitment of Schwann cells into the retina as part of an intrinsic attempt to retard retinal degeneration.

### A Model for Examining Nervous System Repair

Our novel finding, that retinal neurons readily migrate radially out of the retina in response to degeneration of the photoreceptors is of significance, because it has not, to our knowledge, been shown that adult neuronal somata can migrate. This finding out possibilities for studying mechanisms for nervous system repair. Not only did we demonstrate migration of the major inhibitory and excitatory classes of neurons, but we also showed that such neurons may be capable of forming new synapses. Each of these phenomena represent features that would be necessities in studies on the repair of the nervous system—for example, in the spinal cord. Our finding that in human nonneovascular AMD retinas, neurons are frequently present in ectopic locations at the periphery of the degenerative lesion, leads us to believe that such neurons have migrated or are in the process of migrating. This finding that neurons may migrate in an aged human nervous system is an exciting one, and the current possibility of investigating the underpinning events in a rat model may well lead to the possibility of manipulating this phenomenon in other loci for use in a therapeutic context.

We suggest that the Müller cells are the key elements in the migratory process that we observed. Our preliminary data suggest that neuronal migration occurs along the processes of Müller glial cells, which are exuberantly extended radially through gaps in Bruch's membrane, into the overlying choroid.

We conclude that the current rat model provides histologic evidence of extensive remodeling of the retina in response to loss of photoreceptors and that the model may be useful in the study, not only of the dry form of AMD, but also more widely

in studies of neural degeneration and repair in other CNS regions.

### Acknowledgments

The authors thank the Lions Eye Banks (Brisbane and Sydney) for making available human eye tissues and the next of kin and the donors of eye tissues for providing informed consent in making available these tissues for research purposes.

### References

- Chader GJ. Animal models in research on retinal degenerations: past progress and future hope. *Vision Res.* 2002;42:393-399.
- Wang S, Villegas-Perez MP, Vidal-Sanz M, Lund RD. Progressive optic axon dystrophy and vascular changes in *rd* mice. *Invest Ophthalmol Vis Sci.* 2000;41:537-545.
- Lewis GP, Fisher SK. Müller cell outgrowth after retinal detachment: association with cone photoreceptors. *Invest Ophthalmol Vis Sci.* 2000;41:1542-1545.
- Cao W, Tombran-Tink J, Elias R, Sezate S, Mrazek D, McGinnis JF. In vivo protection of photoreceptors from light damage by pigment epithelium-derived factor. *Invest Ophthalmol Vis Sci.* 2001;42:1646-1652.
- Bringmann A, Reichenbach A. Role of Müller cells in retinal degenerations. *Front Biosci.* 2001;6:E72-E92.
- Guidry C, Medeiros NE, Curcio CA. Phenotypic variation of retinal pigment epithelium in age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2002;43:267-273.
- Boulton M, Dayhaw-Barker P. The role of the retinal pigment epithelium: topographical variation and ageing changes. *Eye.* 2001;15:384-389.
- Chang B, Hawes NL, Hurd RE, Davisson MT, Nusinowitz S, Hecklively JR. Retinal degeneration mutants in the mouse. *Vision Res.* 2002;42:517-525.
- He L, Pöhlitz AT, Medrano CJ, Fox DA. Lead and calcium produce rod photoreceptor cell apoptosis by opening the mitochondrial permeability transition pore. *J Biol Chem.* 2000;275:12175-12184.
- Seiler MJ, Liu OL, Cooper NG, Callahan TL, Petry HM, Aramant RB. Selective photoreceptor damage in albino rats using continuous blue light: a protocol useful for retinal degeneration and transplantation research. *Graefes Arch Clin Exp Ophthalmol.* 2000;38:599-607.
- Hetherington L, Benn M, Coffey PJ, Lund RD. Sensory capacity of the royal college of surgeons rat. *Invest Ophthalmol Vis Sci.* 2000;41:3979-3983.
- Akpalaba CO, Oraedu AC, Nwanze EA. Biochemical studies on the effects of continuous light on the albino rat retina. *Exp Eye Res.* 1986;42:1-9.
- Aonuma H, Yamazaki R, Watanabe I. Retinal cell death by light damage. *Jpn J Ophthalmol.* 1999;43:171-179.
- Borges JM, Edward DP, Tso MO. A comparative study of photic injury in four inbred strains of albino rats. *Curr Eye Res.* 1990;9:799-803.
- Mallo G, Goldstein J, Lopez-Costa JJ, Saavedra JP. Expression of c-fos and c-jun in rat retina following protracted illumination. *Brain Res.* 1995;693:196-200.
- Rozañowska M, Jarvis-Evans J, Korytowski W, Boulton ME, Burke JM, Sarna T. Blue light-induced reactivity of retinal age pigment: in vitro generation of oxygen-reactive species. *J Biol Chem.* 1995;270:18825-18830.
- Pauleikhoff D, van Kuijk FJ, Bird AC. Macular pigment and age-related macular degeneration. *Ophthalmology.* 2001;98:511-519.
- Young RW. Solar radiation and age-related macular degeneration. *Surv Ophthalmol.* 1988;32:252-269.
- Mainster MA. Light and macular degeneration: a biophysical and clinical perspective. *Eye* 1987;1:304-310.
- Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Oxidative damage and age-related macular degeneration. *Mol Vis.* 1999;5:32.
- La Cour M, Kiilgaard JF, Nissen MH. Age-related macular degeneration: epidemiology and optimal treatment. *Drugs Aging.* 2002;19:101-133.
- Lai YL, Jacoby RO, Jonas AM. Age-related and light-associated retinal changes in Fischer rats. *Invest Ophthalmol Vis Sci.* 1978;17:634-638.
- Perez J, Perentes E. Light-induced retinopathy in the albino rat in long-term studies: an immunohistochemical and quantitative approach. *Exp Toxicol Pathol.* 1994;46:229-235.
- Reye P, Penfold P, Pow DV. Glyt-1 expression in cultured human Müller cells and intact retinae. *Glia.* 2001;34:311-315.
- Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv Ophthalmol.* 1995;39:367-374.
- Pow DV, Crook DK. Extremely high titre polyclonal antisera against small neurotransmitter molecules: rapid production, characterisation and use in light- and electron-microscopic immunocytochemistry. *J Neurosci Methods.* 1993;48:51-63.
- Gallyas F. Silver staining of myelin by means of physical development. *Neurol Res.* 1979;1:203-209.
- Pow DV, Hendrickson AE. Distribution of the glycine transporter glyt-1 in mammalian and nonmammalian retinae. *Vis Neurosci.* 1999;16:231-239.
- Menger N, Pow DV, Wassle H. Glycinergic amacrine cells of the rat retina. *J Comp Neurol.* 1998;401:34-46.
- Barnett NL, Pow DV. Antisense knockdown of GLAST, a glial glutamate transporter, compromises retinal function. *Invest Ophthalmol Vis Sci.* 2000;41:585-591.
- Friedman B, Hockfield S, Black JA, Woodruff KA, Waxman SG. In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody, Rip. *Glia.* 1989;2:380-390.
- Guymier R, Luthert P, Bird A. Changes in Bruch's membrane and related structures with age. *Prog Retinal Eye Res.* 1999;18:59-90.
- Spraul CW, Lang GE, Grossniklaus HE, Lang GK. Characteristics of drusen and changes in Bruch's membrane in eyes with age-related macular degeneration. *Ophthalmology.* 1998;95:73-79.
- Heriot WJ, Henkind P, Bellhorn RW, Burns MS. Choroidal neovascularization can digest Bruch's membrane: a prior break is not essential. *Ophthalmology.* 1984;91:1603-1608.
- Hayes KC, Carey RE, Schmidt SY. Retinal degeneration associated with taurine deficiency in the cat. *Science.* 1975;188:949-951.
- Rentsch FJ. The ultrastructure of preretinal macular fibrosis. *Graefes Arch Clin Exp Ophthalmol.* 1977;203:171-178.
- Eagle RC Jr. Mechanisms of maculopathy. *Ophthalmology.* 1984;91:613-625.
- Birnbach CD, Jarvelainen M, Possin DE, Milam AH. Histopathology and immunocytochemistry of the neurosensory retina in fundus flavimaculatus. *Ophthalmology.* 1994;101:1211-1219.
- Penfold PL, Wen L, Madigan MC, Gillies MC, King NJ, Provis JM. Triamcinolone acetonide modulates permeability and intercellular adhesion molecule-1 (ICAM-1) expression of the ECV304 cell line: implications for macular degeneration. *Clin Exp Immunol.* 2000;121:458-465.
- Perry VH, Hayes L. Lesion-induced myelin formation in the retina. *J Neurocytol.* 1985;14:297-307.
- Lawrence JM, Sauve Y, Keegan DJ, et al. Schwann cell grafting into the retina of the dystrophic RCS rat limits functional deterioration. *Invest Ophthalmol Vis Sci.* 2000;241:518-528.